Mannitol promotes adherence of an outbreak strain of *Burkholderia multivorans* via an exopolysaccharide-independent mechanism that is associated with upregulation of newly identified fimbrial and afimbrial adhesins

Carmen C. Denman and Alan R. Brown

Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter EX4 4QD, UK

*Burkholderia multivorans*, a member of the *Burkholderia cepacia* complex (Bcc), is an important pathogen of the cystic fibrosis (CF) lung. Mannitol, approved as an inhaled osmolyte therapy for use in CF patients, promotes exopolysaccharide (EPS) production by the Bcc. In the present study, we investigated the role of mannitol-induced EPS in the adherence of *B. multivorans*. We report that mannitol promoted adherence of two representative *B. multivorans* strains. However, whilst this enhanced adherence was largely EPS-dependent in an environmental isolate, it was EPS-independent within a CF outbreak strain, suggesting strain-to-strain variation in adhesins. Genome sequencing of the outbreak strain enabled the identification of two distinct loci encoding putative fimbrial and afimbrial adhesions. The putative fimbriae-encoding locus was found to be widely distributed amongst clinical and environmental *B. multivorans*. In contrast, the locus encoding the putative afimbrial adhesin (of the filamentous haemagglutinin family, FHA) was restricted to clinical isolates. Both loci contributed to biofilm formation and mucin adherence. Furthermore, we report that mannitol promoted expression of both loci, and that the locus encoding the putative FHA-family adhesin is a key determinant of the enhanced adherence observed following growth in mannitol. Our studies provide the first characterization, to our knowledge, of *B. multivorans* adhesins, and in so doing highlight the strain-dependent role of EPS in the Bcc and the difficulties in assigning phenotypic traits to Bcc EPS due to the wider response to mannitol. Our observations also highlight the need to monitor the microbiological effects of inhaled mannitol therapy in Bcc-infected CF patients.

**INTRODUCTION**

Organisms of the *Burkholderia cepacia* complex (Bcc) are problematic opportunistic bacterial pathogens of the cystic fibrosis (CF) lung. Bcc infection is most commonly associated with chronic infection and a gradual deterioration in lung function, and is consistently identified as an independent risk factor for mortality amongst CF patients (Jones et al., 2004; Kalish et al., 2006; Liou et al., 2001). *Burkholderia cenocepacia* and *Burkholderia multivorans* are the most common Bcc species associated with CF respiratory infections, together accounting for approximately 90% of Bcc infections. Until recently, *B. cenocepacia* was the most commonly isolated species, being associated with epidemic spread amongst CF patients. As a consequence, *B. cenocepacia* has received the most research attention, resulting in the identification of numerous putative virulence determinants (recently reviewed by Loutet & Valvano, 2010). However, *B. multivorans* has surpassed *B. cenocepacia* in incidence of respiratory infection amongst CF patients in the USA (LiPuma, 2010) and several European countries (Brisse et al., 2004; Coenye & Vandamme, 2003; Govan et al., 2007; Nørskov-Lauritsen et al., 2010), and in comparison with *B. cenocepacia*, very little is known about the virulence mechanisms and strategies used by *B. multivorans* within the CF host.

Amongst the putative virulence determinants most studied within the Bcc is exopolysaccharide (EPS). When grown on
certain carbon sources (most notably mannitol), Bcc species produce EPS, resulting in a mucoid phenotype (Bartholdson et al., 2008). Whilst clinical B. cenocepacia isolates are frequently non-mucoid (EPS-negative) as a result of mutations within EPS biosynthetic or regulatory pathways, clinical isolates of B. multivorans frequently retain the capacity for EPS production (Zlosnik et al., 2011). EPS production by the Bcc has been associated with bacterial persistence within the lung (Conway et al., 2004), biofilm formation (Cunha et al., 2004) and inhibition of neutrophil activity (Bylund et al., 2006). Additionally, it has recently been proposed that an inverse correlation exists between the quantity of EPS production by Bcc organisms and the rate of decline in CF lung function, and that infections with non-mucoid Bcc are associated with reduced patient survival (Zlosnik et al., 2011). The fact that mannitol is a potent inducer of EPS production by the Bcc has gained clinical relevance following the approval of a dry-powder preparation of mannitol for use as an inhaled osmolyte in CF patients. Whilst this inhaled mannitol therapy has been shown to enhance lung function in CF patients (Bilton et al., 2011; Daviskas et al., 2010; Jaques et al., 2008; Teper et al., 2011), the potential effects on the course of Burkholderia infection within the CF lung is unknown, not least because Bcc-colonization is amongst the listed exclusion criteria that prevents participation in clinical trials.

In the present study, we set out to investigate the role played by mannitol and the associated mucoid phenotype in adherence of B. multivorans. As reported herein, we observed that mannitol promoted adherence, but there existed strain-to-strain variation in the extent to which EPS contributed to this phenotype. Specific adhesins have not previously been described in B. multivorans, and so we sought to identify and characterize the strain-specific adhesins that contributed to these differing phenotypes. Herein we describe the identification and characterization of two distinct adhesin-encoding loci in an outbreak strain of B. multivorans. These loci, which contribute to biofilm formation and mucin adherence, appear specific to B. multivorans and show enhanced expression in the presence of mannitol. These observations provide new insight into B. multivorans biology and highlight the potential microbiological impact of inhaled mannitol therapy in CF patients.

METHODS

**Bacterial strains, plasmids and growth conditions.** Bacteria were routinely cultured at 37 °C in Luria–Bertani (LB) broth (containing 5 g NaCl l⁻¹) supplemented with 1.5 % agar as required. Liquid cultures (10 ml volumes in 30 ml culture vessels) were incubated with shaking at 250 r.p.m. unless stated otherwise. Strains and plasmids are detailed in Table 1. For routine culture, media were supplemented with trimethoprim (100 µg ml⁻¹ for Bcc; 50 µg ml⁻¹ for Escherichia coli), tetracycline (100 µg ml⁻¹ for Bcc; 25 µg ml⁻¹ for E. coli), gentamicin (50 µg ml⁻¹ for Bcc), or kanamycin (25 µg ml⁻¹ for E. coli) as required. Sugar media contained 0.2 % yeast extract (Oxoid) supplemented with either 2 % D-mannitol (for EPS-inducing conditions; YEM media), or 2 % D-mannose for non-EPS-inducing conditions (Sage et al., 1990). All strains employed in this study exhibited similar growth rates within the media used.

**DNA sequencing of B. multivorans C1576 and identification of loci encoding putative adhesins.** Genomic DNA from B. multivorans C1576 was extracted using the PureLink Genomic DNA kit (Invitrogen) and sequenced on an Illumina platform following library preparation using the TruSeq DNA protocol (Illumina). Reads were initially mapped to the B. multivorans ATCC 17616 reference genome, enabling identification of unmapped reads (sequences present in C1576 but absent from ATCC 17616). These unmapped reads were subjected to both de novo assembly and remapping to alternative Bcc reference genomes. Mapping and de novo assembly was performed using CLC Genomics Workbench (CLC bio). Loci encoding putative adhesins were located within the mapped/assembly sequence reads by blastn analysis, and the identity of the putative adhesins to known sequences was assessed by blastn and blastp analysis at NCBI and www.burkholderia.com (Winsor et al., 2008).

**Transcriptome analysis by RNA-seq.** Using the Ribopure-Bacteria kit (Ambion), total RNA was isolated from bacterial cells harvested from agar plates containing 0.2 % yeast extract supplemented with either 2 % mannitol (YEM) or 2 % mannose (two biological replicates per culture condition). Following DNase-treatment (10 U DNase, 1 h), RNA quality was assessed on the Agilent 2100 Bioanalyzer, and only samples with RNA integrity values >7.0 were processed further. Samples (10 µg) of total RNA were subjected to two successive rounds of rRNA depletion using MicrobeExpress (Ambion). Sequencing libraries were prepared from the resulting mRNA-enriched RNA using the TruSeq RNA protocol (Illumina) according to the manufacturer’s instructions prior to sequencing on an Illumina HiSeq2000 platform. Mapping of resulting reads to appropriate reference sequences, calculation of RPKM values (reads per kb of transcript per million mapped reads) and subsequent expression analyses to identify differentially expressed genes [two-group unpaired comparison, with statistical analysis performed on proportions using Baggerley’s test with false discovery rate (FDR)-corrected P-values] were achieved using CLC Genomics Workbench with default parameters.

**Construction of mutants and complemented strains.** Insertional inactivation of target genes was performed using the pGPΩTp suicide vector as described previously (Flannagan et al., 2007). In brief, a 400–500 bp PCR product mapping within the target gene was cloned into pGPΩTp to facilitate homologous recombination and thus insertional inactivation of the target gene. In trans complementation of the resulting mutants was achieved using the previously described pDA17 vector (Flannagan et al., 2008), a broad-host-range plasmid that drives constitutive expression of the cloned gene from the dhfr promoter. All plasmids (for mutagenesis and complementation) were mobilized into the appropriate B. multivorans recipient strain by triparental mating with the helper plasmid, pRK2013 (Figurski & Helinski, 1979). Transformants were selected using gentamicin in conjunction with either trimethoprim (to select for pGPΩTp integrants) or tetracycline (for pDA17-complemented strains). Mutants and complemented strains were confirmed by appropriate PCR validation. All primer sequences are available upon request.

**Adherence to fibronectin.** Bacterial adherence to fibronectin was assessed as described previously (Mil-Homens et al., 2010), with minor modifications. Wells of a 96-well polystyrene plate were coated with 150 µl of fibronectin (10 µg ml⁻¹, Sigma) at 4 °C overnight. Bacteria harvested from fresh overnight LB agar plates were standardized to 10⁶ c.f.u. ml⁻¹ in PBS. Fibronectin-coated wells received either 150 µl bacterial suspension or 150 µl PBS (control).
and were incubated at room temperature for 2 h, after which wells were washed four times with 200 μl PBS. Plates were baked (60 °C, 45 min), prior to staining of adhered cells with filter-sterilized 0.1% (w/v) crystal violet for 15 min. Following staining, wells were washed four times with PBS, before solubilizing bound crystal violet with 95% ethanol (30 min). OD_{570} of solubilized crystal violet was measured on a Bio-Rad Microplate reader. There were 16 replicate wells per strain in each individual experiment.

**Adherence to mucin.** Mucin adherence assays were performed as described previously (Amendolia et al., 2010), with minor modifications. In brief, 50 μl aliquots of filter-sterilized porcine mucin protein (50 μg ml^{-1}, Sigma) were applied to wells of a 96-well polystyrene microtiter plate and incubated overnight at 37 °C. Bacteria were harvested from LB agar plates or mannitol/mannose sugar broths following overnight incubation and standardized to 10⁵ c.f.u. ml⁻¹ in PBS. Mucin-coated wells received either 50 μl bacterial suspension or an equal volume of PBS. Bound bacteria were released from mucin with sterile 0.25% Triton X-100 (Sigma) and enumerated by plating appropriate dilutions (in triplicate) onto LB agar. Six wells were processed per strain in each individual experiment.

**Biofilm assay.** Biofilm formation was assessed using the 96-well plate and accompanying peg-lid of the MBEC Assay device (Innovotech). Bacteria were harvested from LB agar and standardized to 10⁷ c.f.u. ml⁻¹ in tryptone soya broth (TSB) (Oxoid). Wells received either 150 μl bacterial suspension or an equal volume of uninoculated TSB. The peg lid was placed on the plate and the plate incubated at 37 °C for 24 h with shaking (125 r.p.m.). Following 24 h incubation, the peg lid was transferred to a fresh 96-well plate containing pre-warmed TSB and incubated for a further 24 h (37 °C, 125 r.p.m.). The peg lid was then transferred to a 96-well plate containing 200 μl PBS per well and incubated at room temperature for 2 min to remove loosely attached bacteria. The peg lid was baked (60 °C, 20 min) prior to being transferred to a 96-well plate containing 200 μl 0.1% (w/v) crystal violet well per well and incubated for 30 min at room temperature. Three separate wash plates (200 μl PBS per well) rinsed the pegs following staining, and the bound crystal violet was subsequently solubilized with 95% ethanol (30 min) prior to measuring OD_{570}.

**Galleria mellonella infection model.** Infection of larvae was performed largely as described previously (Seed & Dennis, 2008). Larvae were obtained from LiveFoods UK and stored in woodchips at 14 °C prior to use. Using a 25 μl 22g gauge gas-tight Hamilton syringe, larvae were injected in the hindmost proleg with either 10 μl PBS (control) or 10 μl bacterial suspension (10⁷ c.f.u. ml⁻¹). Following injection, larvae were incubated at 37 °C, and their survival was monitored for up to 72 h.

**Dot-blot hybridizations.** Genomic DNA was extracted using the Pure Link Genomic DNA Purification kit (Invitrogen) and was normalized to 85 ng μl⁻¹ in 0.1 M NaOH. Aliquots (3 μl) of the resulting DNA were replica-spotted onto Hybond-N+ membranes (GE Healthcare) and air-dried. Membranes were rinsed (4 × SSC, 5 min; 0.5 × SSC, 5 min) prior to baking (80 °C, 2 h). The PCR DIG Probe Synthesis kit (Roche) was used to generate probes specific for the genes encoding the fimbrial usher protein and HecB-like protein, with the probe sequences corresponding to nucleotides 2515–2940 of JX191919 and nucleotides 12641–13084 of JX191920 respectively. Subsequent pre-hybridization, hybridization, washing and detection were performed using the DIG-Easy Hyb, DIG Wash and Block Buffer.
RESULTS

The contribution of exopolysaccharide to bacterial adhesion is strain-dependent in *B. multivorans*

We sought to determine the role played by mannitol and the associated mucoid phenotype in the adherence of *B. multivorans*. Of the putative EPS biosynthetic gene clusters typically found within the Bcc, the *bce* gene cluster is the most highly conserved (Bartholdson *et al.*, 2008). It has previously been shown that disruption of the gene encoding the BceB glycosyltransferase eliminates EPS biosynthesis and thus the mucoid phenotype in *Burkholderia ambifaria* (Bartholdson *et al.*, 2008). Consequently, to enable the EPS-dependent and EPS-independent effects of mannitol to be determined, we inactivated *bceB* in two representative strains of *B. multivorans*: the environmental isolate *B. multivorans* ATCC 17616 and the CF isolate *B. multivorans* C1576. This CF isolate is the index case of an outbreak within a paediatric CF unit in Glasgow (UK) that was associated with unusually high mortality (Whiteford *et al.*, 1995). This outbreak strain has previously been designated ST-27 by multi-locus sequence typing (MLST) (Baldwin *et al.*, 2005) and will be referred to as such within this study. As anticipated, disruption of *bceB* in the *B. multivorans* strains resulted in a complete loss of EPS production, as judged by visual scoring of mucoidy and quantitative analyses (dry weight and sugar content) of EPS extractions from wild-type and *bceB* mutants following growth on YEM media (data not shown). Disruption of *bceB* did not affect the growth of strains under any culture conditions tested.

We used the mucin adherence assay to assess whether mannitol and/or the associated mucoid phenotype in the adherence of *B. multivorans*. Of the putative EPS biosynthetic gene clusters typically found within the Bcc, the *bce* gene cluster is the most highly conserved (Bartholdson *et al.*, 2008). It has previously been shown that disruption of the gene encoding the BceB glycosyltransferase eliminates EPS biosynthesis and thus the mucoid phenotype in *Burkholderia ambifaria* (Bartholdson *et al.*, 2008). Consequently, to enable the EPS-dependent and EPS-independent effects of mannitol to be determined, we inactivated *bceB* in two representative strains of *B. multivorans*: the environmental isolate *B. multivorans* ATCC 17616 and the CF isolate *B. multivorans* C1576. This CF isolate is the index case of an outbreak within a paediatric CF unit in Glasgow (UK) that was associated with unusually high mortality (Whiteford *et al.*, 1995). This outbreak strain has previously been designated ST-27 by multi-locus sequence typing (MLST) (Baldwin *et al.*, 2005) and will be referred to as such within this study. As anticipated, disruption of *bceB* in the *B. multivorans* strains resulted in a complete loss of EPS production, as judged by visual scoring of mucoidy and quantitative analyses (dry weight and sugar content) of EPS extractions from wild-type and *bceB* mutants following growth on YEM media (data not shown). Disruption of *bceB* did not affect the growth of strains under any culture conditions tested.

We used the mucin adherence assay to assess whether mannitol and/or the associated EPS production influenced adhesion of *B. multivorans* strains ATCC 17616 and C1576. Wild-type and *bceB* mutants of each strain were cultured in the presence of either mannitol (EPS-inducing) or mannose (non-inducing), immediately prior to quantifying adherence to mucin-coated wells. As shown in Fig. 1, within the context of our *in vitro* assay, the environmental ATCC 17616 strain adhered considerably better to mucin than the clinical isolate C1576. However, irrespective of their basal level of adherence, mannitol promoted adherence of both strains to mucin (orthogonal contrast, ATCC 17617: $t=8.9$, d.f. = 51, $P<0.0005$; orthogonal contrast, C1576: $t=4.5$, d.f. = 24, $P<0.0005$). Mannose was chosen as a non-EPS-inducing control as (in contrast to other sugars/sugar alcohols tested) mannitol and mannose supported similar overnight growth of bacteria (when used to supplement 0.2% yeast extract). Mannose has been suggested to act as an anti-adhesive for certain bacterial species, reducing adherence to epithelial cells (Acord *et al.*, 2005). However, it has previously been reported that mannose does not block mucin-binding by *Pseudomonas (Burkholderia) cepacia* (Sajjan & Forstner, 1992). Consistent with this, mucin adherence of our *B. multivorans* strains of interest was not reduced by pre-treatment with mannose (data not shown), indicating that the differential adherence observed between the mannitol- and mannose-grown cultures is due to mannitol promoting adherence, rather than mannose reducing adherence.

Whilst mannitol promoted the adherence of both isolates to mucin, comparison of the wild-type and corresponding EPS-deficient *bceB* mutants revealed differing roles for EPS. As shown in Fig. 1(a), inactivation of *bceB* in ATCC 17616 significantly impaired this enhanced adherence in the presence of mannitol (orthogonal contrast: $t=-6.2$, d.f. = 51, $P<0.0005$), indicating that it was largely EPS-dependent. In contrast, the enhanced adherence of C1576 observed following growth in mannitol was unaffected by the inactivation of *bceB* (orthogonal contrast: $t=-0.2$, d.f. = 24, $P>0.05$; Fig. 1b), indicating that it was independent of EPS status. The differing role for EPS in adherence within these strains is not due to different levels of EPS production, as both wild-type strains yielded similar amounts of EPS (typically 8–11 mg dry weight per Petri dish). These observations led us to sequence the genome of *B. multivorans* C1576 isolate in an attempt to identify genes encoding strain-specific adhesins that may account for this strain-to-strain variation in adherence phenotype.

Identification of loci encoding putative fimbrial and afimbrial adhesins in *B. multivorans* C1576

Illumina genome sequencing of isolate C1576 with subsequent mapping to the ATCC 17616 reference genome allowed us to identify sequences present within C1576 that were absent from ATCC 17616. Analysis of these unmapped reads resulted in the identification of two distinct loci encoding putative fimbrial and afimbrial adhesins (Fig. 2). The sequences of these loci have been deposited in GenBank under the accession numbers JX191919 (fimbrial) and JX191920 (afimbrial). The putative fimbriae-encoding locus is predicted to encode three putative fimbrial proteins, a FimC chaperone protein and a fimbrial usher protein. The nucleotide sequence of the locus is >99% identical to an equivalent fimbriae-encoding locus present in *B. multivorans* CGD1, a recently sequenced isolate from a chronic granulomatous disease (CGD) patient (Varga *et al.*, 2012). In CGD1, the locus is formed by genes BURMUCGD1_3349 to BURMUCGD1_3353. The organization of the locus is identical in the two isolates, and amino acid identity between the corresponding encoded proteins is 99–100%. A similar locus with the same gene organization is observed in two other sequenced CGD *B. multivorans* isolates...
(CGD2 and CGD2M), although the percentage amino acid identity of the encoded proteins compared to those of C1576 is lower (average 83%). Outside of B. multivorans, the predicted fimbrial proteins of B. multivorans C1576 typically exhibit 35–55% amino acid identity with proteins encoded by similar loci in other sequenced Burkholderia or non-Burkholderia species.

The locus encoding the putative afimbrial adhesin of B. multivorans C1576 is depicted in Fig. 2(b). Based on sequence similarity to representative proteins (Figs S1 and S2, available with the online version of this paper), the locus is predicted to encode components of a two-partner secretion (TPS) pathway responsible for the secretion of an adhesin of the filamentous haemagglutinin (FHA) family. The locus encodes two putative TpsA proteins (264 kDa and 68 kDa respectively) that belong to the FHA family of outer-membrane proteins, although only the larger of these two proteins has a mass consistent with the large exoproteins of the TpsA family. The locus is also predicted to encode a single protein of the TpsB family that probably facilitates secretion of the FHA-family adhesin(s). Phylogenetic analysis confirms relatedness of this protein to known TpsB transporter proteins, particularly the HecB protein of Erwinia chrysanthemi (Kim et al., 1998; Rojas et al., 2002) (Fig. S3). Consequently, we propose that the TpsB-family protein of B. multivorans C1576 is a HecB-like protein. Consistent with this, similar phylogenetic analysis of the 264 kDa TpsA family protein of B. multivorans C1576 shows relatedness to the HecA protein of Erwinia chrysanthemi (Fig. S4), although the phylogenetic distances are greater than those observed between the corresponding TpsB proteins. Immediately downstream of the hecB-like gene is a gene encoding a putative PpiC-type peptidyl–prolyl cis–trans isomerase (PPIase) protein, predicted to localize to the cytoplasmic membrane. It is conceivable that this PPIase plays a role in the secretion of the FHA-family protein, as has been described for a periplasmic PPIase of Bordetella pertussis (Hodak et al., 2008).

Analyses of available Burkholderia genomes reveals that the closest FHA-related adhesins to that of B. multivorans facilitate secretion of the FHA-family adhesin(s). Phylogenetic analysis confirms relatedness of this protein to known TpsB transporter proteins, particularly the HecB protein of Erwinia chrysanthemi (Kim et al., 1998; Rojas et al., 2002) (Fig. S3). Consequently, we propose that the TpsB-family protein of B. multivorans C1576 is a HecB-like protein. Consistent with this, similar phylogenetic analysis of the 264 kDa TpsA family protein of B. multivorans C1576 shows relatedness to the HecA protein of Erwinia chrysanthemi (Fig. S4), although the phylogenetic distances are greater than those observed between the corresponding TpsB proteins. Immediately downstream of the hecB-like gene is a gene encoding a putative PpiC-type peptidyl–prolyl cis–trans isomerase (PPIase) protein, predicted to localize to the cytoplasmic membrane. It is conceivable that this PPIase plays a role in the secretion of the FHA-family protein, as has been described for a periplasmic PPIase of Bordetella pertussis (Hodak et al., 2008).

Analyses of available Burkholderia genomes reveals that the closest FHA-related adhesins to that of B. multivorans facilitate secretion of the FHA-family adhesin(s). Phylogenetic analysis confirms relatedness of this protein to known TpsB transporter proteins, particularly the HecB protein of Erwinia chrysanthemi (Kim et al., 1998; Rojas et al., 2002) (Fig. S3). Consequently, we propose that the TpsB-family protein of B. multivorans C1576 is a HecB-like protein. Consistent with this, similar phylogenetic analysis of the 264 kDa TpsA family protein of B. multivorans C1576 shows relatedness to the HecA protein of Erwinia chrysanthemi (Fig. S4), although the phylogenetic distances are greater than those observed between the corresponding TpsB proteins. Immediately downstream of the hecB-like gene is a gene encoding a putative PpiC-type peptidyl–prolyl cis–trans isomerase (PPIase) protein, predicted to localize to the cytoplasmic membrane. It is conceivable that this PPIase plays a role in the secretion of the FHA-family protein, as has been described for a periplasmic PPIase of Bordetella pertussis (Hodak et al., 2008).

Analyses of available Burkholderia genomes reveals that the closest FHA-related adhesins to that of B. multivorans facilitate secretion of the FHA-family adhesin(s). Phylogenetic analysis confirms relatedness of this protein to known TpsB transporter proteins, particularly the HecB protein of Erwinia chrysanthemi (Kim et al., 1998; Rojas et al., 2002) (Fig. S3). Consequently, we propose that the TpsB-family protein of B. multivorans C1576 is a HecB-like protein. Consistent with this, similar phylogenetic analysis of the 264 kDa TpsA family protein of B. multivorans C1576 shows relatedness to the HecA protein of Erwinia chrysanthemi (Fig. S4), although the phylogenetic distances are greater than those observed between the corresponding TpsB proteins. Immediately downstream of the hecB-like gene is a gene encoding a putative PpiC-type peptidyl–prolyl cis–trans isomerase (PPIase) protein, predicted to localize to the cytoplasmic membrane. It is conceivable that this PPIase plays a role in the secretion of the FHA-family protein, as has been described for a periplasmic PPIase of Bordetella pertussis (Hodak et al., 2008).

Analyses of available Burkholderia genomes reveals that the closest FHA-related adhesins to that of B. multivorans
C1576 are found in the environmental isolate *B. ambifaria* MC40-6 (approximately 83% amino acid identity between corresponding proteins) and *Burkholderia phymatum* STM815 (approximately 65% amino acid identity between corresponding proteins). Amino acid identity to related proteins of non-*Burkholderia* species (including the prototypic FHA/FhaC of *Bordetella pertussis* and the HecA/HecB of *Erwinia chrysanthemi*) is approximately 28–30%.

**The loci encoding the putative adhesins of *B. multivorans* C1576 differ in their strain distribution**

The analysis of available genome sequences referred to above suggested that the two loci were not widely distributed within the Bcc and wider *Burkholderia* genus. Using dot-blot hybridization, we assessed the distribution of each locus (based on the presence or absence of a representative gene for each) within a wider panel of *Burkholderia* isolates, with a particular focus on clinical *B. multivorans* isolates. Genomic DNA was isolated from a total of 97 Bcc isolates, comprising 83 clinical *B. multivorans* isolates (including 26 representatives of strain ST-27), six environmental *B. multivorans* isolates and representatives of other Bcc species (for strain details, see Table S1). In the course of verifying the *B. multivorans* isolates belonging to the ST-27 outbreak strain, MLST analysis revealed that whilst all shared an identical MLST type, this differed by a single nucleotide within the *lepA* locus from the previously published MLST type for *B. multivorans* C1576 (Baldwin et al., 2005). The corrected MLST profile for the ST-27 outbreak strain (represented by the index case C1576) is thus: *atpD* 13, *gltB* 7, *gyrB* 6, *recA* 10, *lepA* 224, *phaC* 42 and *trpB* 6.

Genomic DNA from the assembled strain panel was probed for the presence of genes encoding the putative fimbrial usher and the HecB-like proteins. Based on the presence of these representative genes, results (summarized in Table 2 and detailed in Table S1) reveal that the locus encoding the putative fimbrial proteins is widely distributed amongst both clinical and environmental *B. multivorans* isolates, being observed in 76 of 89 isolates tested. In contrast, the locus encoding the FHA-family adhesin is more restricted in distribution, being limited to clinical isolates and particularly the ST-27 outbreak strain (occurring in 100% of ST-27 isolates, compared with <9% of non-ST-27 isolates). None of the non-*B. multivorans* isolates within the strain panel harboured either gene.

**Fimbrial and FHA-family adhesins of *B. multivorans* C1576 contribute to mucin adherence and biofilm formation**

We next sought to evaluate the role of these putative adhesins in adherence and biofilm formation. Individual mutants were generated by insertional inactivation of the genes encoding the putative HecB-like and fimbrial usher proteins (resulting in strains hecB CR and fim CR respectively, Table 1). Both mutants were complemented in trans using the pDA17 constitutive expression vector. Additionally, RT-PCR analysis confirmed that genes flanking the inactivated genes within each of the mutants were still expressed (data not shown).

To evaluate the role of the adhesins in abiotic adherence, we assessed the ability of the strains to adhere to mucin and to the extracellular matrix protein fibronectin. Neither mutant exhibited a significant reduction in fibronectin binding (orthogonal contrast: *t* = −0.3, d.f. = 25, *P* > 0.05). In contrast, both adhesin mutants were significantly reduced in their adherence to mucin relative to the wild-type strain (orthogonal contrast: *t* = −6.7, d.f. = 85, *P* < 0.0005; Fig. 3a). Appropriate complementation of the mutants (fim CO and hecB CO) partially restored mucin adherence (*P* < 0.05 relative to the corresponding mutant strain), albeit not to wild-type levels (Fig. 3a). This partial complementation has been observed previously with the pDA17 vector, including in a recent study of an adhesin-like gene of *B. cenocepacia* (Mil-Homens et al., 2010), and we believe it reflects suboptimal gene dosage from the complementation vector.

We assessed the ability of all strains to form biofilm using the MBEC biofilm device (Innovotech). As shown in Fig. 3(b), both mutant strains (fim CR and hecB CR) exhibited significantly impaired biofilm formation relative to wild-type C1576 (orthogonal contrast: *t* = −6.4, d.f. = 115, *P* < 0.0005), indicating that both adhesins are important for biofilm formation. Appropriate complementation of both mutants fully restored biofilm formation (Fig. 3b).

Finally, to investigate the role of these adhesins during infection, all strains were assessed within the *Galleria mellonella* infection model, a model that has been used previously to investigate the role of *Burkholderia* adhesins (Mil-Homens et al., 2010). Neither mutant exhibited altered larval killing compared with wild-type C1576, with all strains killing 100% of larvae within 72 h at an inoculum of approximately 10^6 c.f.u. (data not shown). Larvae that received injections of PBS exhibited 100% survival for the duration of the experiment. Therefore, individually, neither the putative fimbrial usher protein

**Table 2. Distribution of the genes encoding the putative HecB-like and fimbrial usher proteins amongst clinical and environmental *B. multivorans* isolates, as determined by dot-blot analysis**

Clinical isolates are subdivided into ST-27 and non-ST-27.

<table>
<thead>
<tr>
<th></th>
<th>Clinical (ST-27)</th>
<th>Clinical (non-ST-27)</th>
<th>Environmental</th>
</tr>
</thead>
<tbody>
<tr>
<td>hecB-like</td>
<td>26/26</td>
<td>5/57</td>
<td>0/6</td>
</tr>
<tr>
<td>fim</td>
<td>26/26</td>
<td>47/57</td>
<td>3/6</td>
</tr>
</tbody>
</table>
nor the HecB-like protein is required for full virulence in the *Galleria* model.

**Mannitol promotes expression of the putative fimbrial and FHA-family adhesins**

Having shown that growth of C1576 in mannitol promoted adherence to mucin in an EPS-independent manner (Fig. 1b), we assessed whether growth in mannitol increased the expression of either of the putative adhesin-encoding loci characterized above. As part of a wider on-going study of the genome-wide transcriptional response of *B. multivorans* to mannitol, we have performed RNA-seq analysis on the Illumina platform. The full dataset arising from these studies will be published elsewhere. However, analysis of this transcriptome dataset reveals that growth in mannitol promotes expression of both loci encoding the putative fimbrial and afimbrial adhesins (Fig. 4). This is particularly the case for the putative fimbriae-encoding locus, every gene of which is significantly upregulated by growth in mannitol. We also observed significant upregulation of the cepacian biosynthetic gene clusters *bce-I* and *bce-II* (Ferreira et al., 2010) following growth in YEM (Table S2), consistent with the observed induction of EPS biosynthesis on YEM and the previous report that growth on YEM upregulates *bceE* expression in *B. ambifaria* AMMD (Bartholdson et al., 2008).

**The putative FHA-family adhesin of *B. multivorans* C1576 contributes to the enhanced adherence following growth in mannitol**

Having shown upregulation of both the fimbrial and afimbrial adhesins in response to mannitol, we assessed the extent to which the individual adhesins contribute to the mannitol-promoted adherence phenotype observed previously (Fig. 1b). The wild-type and adhesin mutants were cultured in mannose- or mannitol-supplemented broth prior to the mucin adherence assay. Relative to wild-type, both mutants showed impaired mucin adherence following growth in mannose-containing media (orthogonal contrast: \( t = -2.2, \ d.f. = 112, \ P < 0.05 \)), consistent with the results presented in Fig. 3(a) and consistent with a general defect in adherence following inactivation of either adhesin. Despite this lower basal level of adherence, the adherence of the *fim* mutant was still elevated five- to sixfold following growth in mannitol (orthogonal contrast: \( t = -16.6, \ d.f. = 112, \ P < 0.0005 \)). This is consistent with observations of the wild-type, and suggests that the putative fimbrial adhesin does not play a significant role in the enhanced mucin adherence that is induced by mannitol. In contrast, the adherence of the *hecB* CR strain is not enhanced following growth in mannitol (orthogonal contrast: \( t = -0.7, \ d.f. = 112, \ P > 0.05 \)), indicating that the putative FHA-family adhesin contributes to the mannitol-induced mucin adherence.

**DISCUSSION**

In this study we have shown that mannitol can promote adherence of *B. multivorans* in an EPS-independent manner that is associated with the upregulation of genes encoding putative fimbrial and FHA-family adhesins. Whilst it is the fimbrial locus that is most significantly elevated following growth in mannitol, it is the locus encoding the putative FHA-family adhesin that appears to be a key determinant of the enhanced adherence. Although perhaps unexpected, this may reflect the fact that the *hecB* gene is predicted to encode the transporter for the FHA-family adhesin.
adhesin rather than the adhesin itself, and thus hecB expression is not a reliable indicator of the amount of mature adhesin on the cell surface.

Whilst adhesins have been described in other members of the Bcc and within the wider Burkholderia genus (Balder et al., 2010; Mil-Homens et al., 2010; Mil-Homens & Fialho, 2012; Urban et al., 2005), the present study represents the first investigation, to our knowledge, of putative fimbrial and FHA-family adhesins within the Bcc and the first characterization of any specific adhesins within B. multivorans. Whilst fimbrial and FHA-family adhesins are common within the Burkholderia genus, there is considerable diversity at the amino acid level between the adhesins of different species. Such sequence variation within adhesins can profoundly alter their binding specificities and affinities, thus affecting host tropism and capacity for virulence (Sokurenko et al., 1998; Weissman et al., 2006). Burkholderia species are an extremely versatile group of organisms, having been reported as human and animal pathogens, plant pathogens, plant growth promoters and endosymbions of insects and fungi (for review, see Vial et al., 2011). The role played by species-specific and strain-specific adhesins in conferring such diverse host- and niche-adaptations of Burkholderia remains to be elucidated. Within the present study, we observed that the environmental isolate (ATCC 17616) adhered significantly better to mucin than the clinical isolate (C1576), and further studies are required to identify the molecular basis for this. A heightened capacity for adherence to mucin may promote colonization of the airways. Epidemiological data indicate that the majority of new cases of B. multivorans infection in CF patients are due to acquisition from environmental sources (Brise et al., 2004; Coenye & Vandamme, 2003; Govan et al., 2007; Norskov-Lauritsen et al., 2010), and it is notable that CF isolates of B. multivorans that share the same MLST profile as the environmental ATCC 17616 isolate have been recovered from CF patients (Baldwin et al., 2008).

In contrast to the widely distributed fimbrial-encoding locus, it is striking that the locus encoding the putative FHA-family adhesin reported herein was found only in clinical isolates. Clearly further environmental isolates need to be studied to test this association more rigorously – the low number of environmental B. multivorans included in the assembled strain panel reflects the infrequent isolation of this species from the natural environment (Baldwin et al., 2008). Furthermore, all but two of the clinical isolates detailed in Table S1 that were found to harbour the hecB-like gene (representative of the FHA-encoding locus) belong to sequence types known to be associated with patient-to-patient transmission, notably ST-27, ST-25 and ST-179 (Baldwin et al., 2008). Putative transmissibility factors have been identified in other Bcc species (Clode et al., 2000), and whilst their predictive value has been questioned (Govan et al., 2007), our observations justify further studies to investigate the strength of the association of the FHA-encoding locus with transmissible strains and its potential as a marker for transmissibility amongst clinical isolates of B. multivorans.

The rationale for this study of adhesins within the B. multivorans ST-27 strain stemmed from our observations that whilst mannitol promoted bacterial adherence to mucin, the role played by EPS was strain-dependent. There remains considerable debate about the significance of EPS production as a Bcc virulence factor, fuelled by the fact that arguably one of the most virulent Bcc strains (B. cenocepacia ET-12) does not produce EPS and is consistently non-mucoid (Bartholdson et al., 2008; Zlosnik et al., 2008). Our observations from the present study emphasize...
the difficulty in establishing the role of EPS in virulence, as
the growth conditions commonly used to induce and study
EPS (the mannitol-containing media, YEM) clearly elicit a
response within the bacterium that is wider than the EPS
biosynthetic pathway. Consequently, attempts to identify
the role of EPS through phenotypic observations of whole
organisms are prone to being skewed by the wider EPS-
independent response to mannitol, potentially resulting in
phenotypic traits being wrongly assigned to the mucoid
phenotype. We suggest that the approach undertaken in
the present study (parallel studies of the wild-type and an
isogenic EPS-deficient mutant) is best-suited for identifi-
ing phenotypes truly associated with EPS production,
although clearly such an approach depends on having
sufficient knowledge of the genetic basis of EPS biosyn-
thesis. Our observation that EPS plays a significant role in
the mucin adherence of ATCC 17616 but not the C1576
strain also highlights the multi-factorial aspect of Bcc
virulence. The contribution of individual virulence deter-
minants can vary from strain to strain and can be
influenced by the presence or absence of other com-
plementary factors. With specific reference to EPS, one
consequence of this is that the biological significance of
the documented mucoid to non-mucoid transition that is
frequently observed in sequential Bcc isolates from
individual CF patients (Zlosnik et al., 2008) may be
strain-dependent. This may also be influenced by differ-
ences within the EPS itself, as the differing roles attributed
to the EPS of the two strains studied herein may reflect
differences in EPS composition and structure. Whilst
beyond the scope of the current study, there is clearly
merit in performing a structure–function analysis of
Burkholderia EPS.

During early pilot studies of inhaled mannitol therapy, it
was acknowledged that the ability to utilize mannitol as a
carbon and energy source was common amongst both Bcc
and Pseudomonas aeruginosa (the dominant CF pathogen)
(Robinson et al., 1999). However, it was considered
unlikely that this would significantly affect bacterial burden
in the lung due to the abundance of alternative nutrient
sources within respiratory secretions. This appears to have
been borne out by clinical trials that have found no
difference in sputum microbiology between treatment and
control groups (Jaques et al., 2008; Teper et al., 2011).
However, the fact that mannitol promotes EPS production,
together with our observations that mannitol promotes
expression of adhesin-encoding loci that contribute to
abiotic adherence and biofilm formation, indicate that
administration of mannitol is likely to have profound
phenotypic consequences on Bcc within the lung.
Furthermore, by enhancing expression of the adhesins, it
is conceivable that mannitol may promote the initial
colonization of the airways with Bcc. The full effects of
mannitol on relevant virulence traits of the Bcc (and
indeed other microbial species commonly found in the CF
lung) have yet to be established. However, based solely on
observations to date, the phenotypic effect of mannitol on
the Bcc is unequivocal, and close microbiological monitor-
ing of patients receiving inhaled mannitol therapy would
appear prudent.

ACKNOWLEDGEMENTS

This work was supported by the Cystic Fibrosis Trust and a PhD
studentship from the University of Exeter. The authors have no
conflict of interest to declare. We are grateful to Konrad Paszkiewicz,
Karen Moore, Audrey Farbos and Alex Moorhouse from the
University of Exeter Sequencing Service; Eshwar Mahenthiralingam
(Cardiff University) and Cathy Doherty and John Govan (University
of Edinburgh) for provision of isolates and related strain information;
Miguel Valvano (University of Western Ontario) for provision of
vectors for mutagenesis and complementation; and James Cresswell
(University of Exeter) for helpful discussion on statistical methods.

REFERENCES

for quantifying inhibition of bacterial adhesion to eukaryotic cells.
Ammendolia, M. G., Bertuccini, L., Iosi, F., Minelli, F., Berluti, F.,
Valenti, P. & Superti, F. (2010). Bovine lactoferrin interacts with cable
Balder, R., Lipski, S., Lazarus, J. J., Grose, W., Wooten, R. M., Hogan,
Burkholderia mallei and Burkholderia pseudomallei adhesins for
human respiratory epithelial cells. BMC Microbiol 10, 250.
Baldwin, A., Mahenthiralingam, E., Thickett, K. M., Honeybourne, D.,
Maiden, M. C., Govan, J. R., Speert, D. P., Lipuma, J. J., Vandamme, P.
& Dowson, C. G. (2005). Multilocus sequence typing scheme that
provides both species and strain differentiation for the Burkholderia
Baldwin, A., Mahenthiralingam, E., Drevenek, P., Pope, C., Waine,
D. J., Henry, D. A., Speert, D. P., Carter, P., Vandamme, P. & other
authors (2008). Elucidating global epidemiology of Burkholderia
multivorans in cases of cystic fibrosis by multilocus sequence typing.
Bartholdson, S. J., Brown, A. R., Mewburn, B. R., Clarke, D. J., Fry,
alcohol induced exopolysaccharide biosynthesis in the Burkholderia
cepacia complex. Microbiology 154, 2521–2521.
Bilton, D., Robinson, P., Cooper, P., Gallagher, C. G., Kolbe, J., Fox,
Inhaled dry powder mannitol in cystic fibrosis: an efficacy and safety
Brisse, S., Cordevant, C., Vandamme, P., Bidet, P., Loukil, C.,
and ribotype diversity of Burkholderia cepacia complex isolates from
French patients with cystic fibrosis. J Clin Microbiol 42, 4824–
4827.
(2006). Exopolysaccharides from Burkholderia cenocepacia inhibit
neutrophil chemotaxis and scavenger reactive oxygen species. J Biol
Chem 281, 2526–2532.
Distribution of genes encoding putative transmissibility factors
among epidemic and nonepidemic strains of Burkholderia cepacia
from cystic fibrosis patients in the United Kingdom. J Clin Microbiol
38, 1763–1766.


rate of structural mutations in fimbrial adhesins of extraintestinal pathogenic Escherichia coli. Mol Microbiol 59, 975–988.


